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Prokupek, Adrianne; Hoffmann, Federico G.; Eyun, Seong-il; Moriyama, Etsuko N.; Zhou, Min; and Harshman, Lawrence G., "An Evolutionary Expressed Sequence Tag Analysis of *Drosophila* Spermatheca Genes" (2008). *Papers in Genetics*. 7.

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An Evolutionary Expressed Sequence Tag Analysis of *Drosophila* Spermatheca Genes

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Abstract

This study investigates genes enriched for expression in the spermatheca, the long-term sperm storage organ (SSO) of female *Drosophila*. SSO genes are likely to play an important role in processes of sexual selection such as sperm competition and cryptic female choice. Although there is keen interest in the mechanisms of sexual selection at the molecular level, very little is known about the female genes that are involved. In the present study, a high proportion of genes enriched for expression in the spermatheca are evolving rapidly. Most of the rapidly evolving genes are proteases and genes of unknown function that could play a specialized role in the spermatheca. A high percentage of the rapidly evolving genes have secretion signals and thus could encode proteins that directly interact with ejaculate proteins and coevolve with them. In addition to identifying rapidly evolving genes, the present study documents categories of genes that could play a role in spermatheca function such as storing, maintaining, and utilizing sperm. In general, candidate genes discovered in this study could play a key role in sperm competition, cryptic female choice of sperm, and sexually antagonistic coevolution, and ultimately speciation.

Keywords: coevolution, molecular evolution, sexual selection, speciation, sperm storage

Darwin (1871) defined sexual selection as the advantage some individuals have over others of the same sex in relation to reproduction. This type of selection can result in the evolution of conspicuous traits such as extravagant secondary sexual characteristics in some species or, more subtly, may be working on a molecular level through the evolution of proteins involved in reproductive processes such as sperm competition or female sperm choice. Rapidly evolving reproductive proteins are likely involved in sexual selection, playing specific roles in inter (between), intra (within) sex competitions, or a combination of both. One major area of deficit in these studies is in the identification and classification of female reproductive proteins, especially those proteins interacting directly with male seminal products (e.g., sperm storage proteins). Important female reproductive proteins are likely to be found

in organs dedicated to the storage of sperm. Sperm storage organs (SSOs) are found in the females of a variety of animal taxa and function in the retention, maintenance, and use of sperm after mating has occurred. The present study focuses on one of the two types of SSOs present in *Drosophila*, the spermathecae, which is the long-term SSO.

Although spermathecae are commonly found in a range of invertebrate and vertebrate taxa (Eberhard 1996), the function of proteins and other macromolecules associated with this organ are understudied. An exception is social insects—bees and ants—in which the spermatheca are known to be important for long-term sperm storage (Wheeler and Kruttsch 1994; Weirich *et al.* 2002; Collins *et al.* 2004; Collins *et al.* 2006). Reproductive proteins associated with female SSOs (such as spermathecae) are candidates to play important roles in evolutionary phenomena

such as sperm competition, female sperm choice, sexually antagonistic coevolution, and consequently in speciation. In spite of their potential importance, little is known about how they function and even less about the evolution of genes associated with these organs. Identification of female proteins expressed within the spermathecae is a vital step in the development of a comprehensive understanding of the role of SSOs in evolution.

Specific genes and proteins known to play a role in sperm competition have, thus far, only been identified in males. In *Drosophila melanogaster* second male sperm precedence (P2) is due, in part, to a nonsperm component of the ejaculate (Harshman and Prout 1994). It is now established that male accessory gland proteins play a role in sperm competition in *Drosophila* (Ravi-Ram and Wolfner 2007). Allelic variation in male accessory gland protein genes has been associated with the differential fertilization success of both the first male to mate with a female and the second male to mate with the female (Clark *et al.* 1995; Fiumera *et al.* 2005, 2007). Genetic studies reveal that female processes have a major effect on the outcome of sperm competition in *D. melanogaster* (Clark and Begun 1998; Clark *et al.* 1999), but the specific female genes that have these effects are not known.

Studies of conspecific sperm precedence have provided clues about the importance of male and female reproductive proteins in sperm competition (Coyne and Orr 2004). Conspecific sperm precedence occurs when a female mated to both conspecific and heterospecific males, regardless of the mating order, preferentially produces conspecific rather than hybrid offspring (Howard 1999). This phenomenon has been observed in a diverse range of taxa including flour beetles, sea urchins, *Drosophila*, rabbits, and several plant species (reviewed in Howard 1999; Howard *et al.* 2008). In *D. melanogaster*, conspecific sperm precedence can involve the incapacitation of sperm of the first male to mate by the seminal fluid of the second male to mate (Price 1997; Price *et al.* 1999). In this species, heterospecific sperm are not displaced from the female SSOs by the seminal fluid of the second male to mate. Thus, sperm precedence may be due to interactions between male seminal fluid and female reproductive proteins in SSOs. In *D. mauritiana*, stored heterospecific sperm are rapidly lost from SSOs (Price *et al.* 2001), presumably due to improper storage. Female SSOs could play a major role in conspecific sperm precedence, or preclude fertilization by heterospecific sperm.

Rapid evolution of reproductive proteins has been documented in protists, fungi, plants, and animals (Clark *et al.* 2006) and in both male and female gametic proteins (Swanson and Vacquier 2002; Galindo *et al.* 2003). For example, in sea urchins male sperm bindin evolves rapidly (Palumbi 1999) as does the bindin receptor on the egg (Palumbi 1999; Kamei *et al.* 2000). In mammals, egg coat zona pellucida glycoproteins and several sperm proteins evolve rapidly and exhibit the molecular signature of positive (adaptive) selection (Swanson *et al.* 2001b, 2003). Rapidly evolving *Drosophila* male accessory gland

proteins (Acps) have been a focus of molecular population genetic and molecular evolution studies. The average rate of sequence divergence of *D. melanogaster* Acps is approximately twice that of nonreproductive proteins (Begun *et al.* 2000; Swanson *et al.* 2001a; Wagstaff and Begun 2004; Mueller *et al.* 2005). By contrast, female reproductive genes are understudied in *Drosophila*, but the signature of positive selection has been revealed by evolutionary expressed sequence tag (EST) studies using the lower reproductive tract of both *D. simulans* (Swanson *et al.* 2004) and *D. arizonae* (Kelleher *et al.* 2007). These studies did not investigate the rate of evolution of a broad sample of genes from a specific organ as was done in the present study. The present study is the first molecular evolutionary study of genes sampled from a specific female SSO in any species.

Drosophila species typically have two types of organs dedicated to sperm storage (Fowler 1973; Pitnick *et al.* 1999). The seminal receptacle contains the majority (65–80%) of the sperm (Lefevre and Jonsson 1962; Neubaum and Wolfner 1999), whereas a pair of spermathecae are the site of long-term storage. Sperm are stored in the spermathecal lumen, which receives proteins of unknown function from surrounding secretory epithelial cells (Filosi and Perotti 1975). Evolutionary interactions have been identified between sperm and SSOs. For example, evolutionary changes in sperm length resulted in corresponding changes in the length of the seminal receptacle (Miller and Pitnick 2002, 2003). One rationale for investigating genes in the spermatheca is that rapidly evolving genes in this SSO might coevolve with rapidly evolving *Drosophila* Acps.

The present study is an evolutionary EST investigation of genes enriched for expression in *Drosophila* spermathecae. The focus is on spermathecae because they secrete proteins into the sperm storage lumen that could interact with male proteins in the female (Acps and sperm proteins). Rapidly evolving proteins in the spermatheca are prime candidates to play an important role in female-ejaculate interactions. The results suggest that a high proportion of spermathecal proteins evolve rapidly. Such proteins include those with secretion signals and thus are capable of directly interacting with male reproductive proteins in this SSO. Female-ejaculate interactions are thought to mediate key features of sperm storage and important evolutionary phenomena.

Methods and Materials

cDNA Library Preparation and Dna Sequence Generation

RNA was isolated from both spermathecae, including the spermathecal ducts, dissected from 250 *D. simulans* females. The females were held as virgins until the fourth day of adult life when each was paired with a single male. Dissection occurred 3 h after mating was observed. Total RNA was isolated from spermathecae us-

ing the TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was also purified from female whole bodies minus spermathecae to be used as the driver in subtractive hybridization. cDNA was generated from total RNA using the SMART (Simple Modular Architecture Research Tool) approach (Zhu *et al.* 2001). Subtractive hybridization was performed and a cDNA library was generated (Evrogen, Moscow, Russia) using the suppressive subtraction hybridization (SSH) method in both directions (tester vs. driver and driver vs. tester) (Diatchenko *et al.* 1996, 1999). An aliquot of the library was plated and 384 colonies were used for DNA template generation by rolling circle amplification using TempliPhi (Amersham Biosciences, Piscataway, NJ). Three hundred and eighty-three DNA sequences were generated using the MegaBACE 400 automated DNA sequencer (Amersham Biosciences). Vector sequences were masked using the CAP3 program (Huang and Madan 1999).

Identification of Genes Expressed in Spermathecae

To ensure that we found all the genes represented by the *D. simulans* spermathecae EST (expressed sequence tags) sequences, we queried both the *D. simulans* and *D. melanogaster* genomes as the latter is more complete. Each of the 383 *D. simulans* ESTs was used as a query in a blastn DNA similarity search (Altschul *et al.* 1990) conducted against the entire CDS sets of *D. simulans* and *D. melanogaster*. We excluded those sequences with similarities of 80% or lower, and those with expected (E) values greater than 0.01 (139 sequences were excluded).

Ortholog Identification

Using each of the *D. simulans* CDSs obtained above as a query, a blastp protein similarity search (Altschul *et al.* 1990) was performed to identify ortholog candidates from five additional *Drosophila* genomes (*D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, and *D. pseudoobscura*). The Comparative Analysis Freeze 1 (CAF1) genomic sequences of the *Drosophila* species were downloaded from the AAWiki website (<http://rana.lbl.gov/Drosophila>) of the 12 *Drosophila* genome project. The entire set of coding sequences of *D. melanogaster* was obtained from FlyBase (Release 5.1; <http://flybase.org>). The top hit from each species was then used as a query and a reciprocal blastp search was performed against the entire *D. simulans* CDS set to confirm the orthologous relationships. When multiple sequences were identified with almost identical lowest E-values, all were used as the queries for the reciprocal blastp search. After examining the results of the reciprocal search, ortholog candidates from each species were identified for each of the *D. simulans* genes. To determine the presence or absence of possible distant orthologs in other species, reciprocal blast was performed against an additional five (more-distantly related) *Drosophila* genomes (*D. persimilis*, *D. willistoni*, *D. mojavensis*,

D. virilis, and *D. grimshawi*). In addition to blastp, tblastn against these DNA scaffolds was also used.

Species-Specific Duplications

Our orthologous gene set was compared to the list of homologs provided by the 12 *Drosophila* genome project (http://rana.lbl.gov/~venky/AAA/freeze_20061030/protein_coding_gene). As the list provided by the genome project identified only homolog candidates regardless of whether a gene was an ortholog or paralog, we confirmed that all of our ortholog candidates were included among their homolog candidates. If two or more genes were identified as the top hits with almost identical E-values, then these genes were analyzed as possible duplicates by further investigation including DNA and protein phylogenetic analysis to identify paralog/ortholog relationships.

Reconstruction of Multiple Alignments from Orthologous Gene Sets

We first reconstructed protein alignments using MUSCLE (Edgar 2004), and each alignment was adjusted manually. Protein alignments were reverse translated to nucleotide alignments based on their nucleotide sequences using the protal2dna web server (<http://bioweb.pasteur.fr/seqanal/interfaces/protal2dna.html>). Each nucleotide alignment was again adjusted manually. Finally, the nucleotide alignments were translated to protein alignments for confirmation. The final nucleotide alignments were used for molecular evolutionary analyses.

Evolutionary Analyses

The relative contribution of nonsynonymous (d_N) and synonymous (d_S) changes to the patterns of nucleotide variation was compared using the codon-based maximum-likelihood framework described by Goldman and Yang (1994) implemented in PAML version 3.15 (Yang *et al.* 2000). A pairwise comparison was performed between *D. simulans* and *D. melanogaster*. The likelihood of d_N being higher than d_S was evaluated by comparing a model in which d_N and d_S were estimated as free parameters (L_1) to a model in which d_N equals d_S (L_0). The two models were compared in a likelihood-ratio test with one degree of freedom. Historically, d_N/d_S ratios that exceeded 1.0 were considered to be indicative of positive selection, but recent studies, such as Swanson *et al.* (2004), argue that this ratio is conservative, especially for the identification of candidate genes. Lowering the d_N/d_S ratio to 0.5 was found to be reasonable for the identification of candidate genes to undergo further investigation into the forces of selection (Swanson *et al.* 2004). The d_N/d_S ratios were also estimated by using the branch model of PAML. This model allows the d_N/d_S ratios to vary among branches in a given phylogeny and is useful in detecting positive selection acting on particular lineages (Yang 1998).

Variation in the d_N/d_S ratio among sites was also explored using the tree-based models described by Yang *et al.* (2000) based on the alignment of *D. simulans*, *D. melanogaster*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae* and *D. pseudoobscura* orthologs. PAML was run using the maximum number of orthologs possible; a minimum of four orthologs were used to circumvent problems caused by model convergence. The inclusion of *D. pseudoobscura* could lead to an overestimation of d_N/d_S due to a saturation of d_S obtained between *D. pseudoobscura* and other species. Therefore, the analysis was done both including and excluding *D. pseudoobscura*. Additionally, to ensure that possible incomplete lineage sorting reported in the *melanogaster* subgroup (Pollard *et al.* 2006; Wong *et al.* 2008) did not affect the outcome of our analysis, the analysis was done using three six-species trees varying the phylogenetic placement of *D. erecta* and *D. yakuba*. The assumptions of the models and test statistics are briefly described in Results, for a full description see Yang *et al.* (2000).

Transmembrane and Signal Peptide Prediction and Functional Domain Detection

Protein sequences from *D. melanogaster* orthologs were used for motif prediction. Transmembrane (TM) region prediction was conducted using two programs: HMMTOP version 2.0 (Tusnady and Simon 2001) and Phobius (Kall *et al.* 2004). Both methods use hidden Markov models for predicting the transmembrane topology. Phobius combines TM prediction and signal peptide prediction to identify signal peptides from N-terminal regions, often misidentified as a TM region by these prediction methods. We list a protein as having a transmembrane domain if both HMMTOP and Phobius predicted TM regions, or if one program predicted more than one TM region. For signal peptide prediction, we used TargetP version 1.1 (Emanuelsson *et al.* 2007) in addition to Phobius. The TargetP program ranks support for the signal peptides. Only the genes in the highest class of support, which were also identified as having signal peptides by Phobius, were listed as having signal peptides.

Functional Categories

All genes were subject to conserved domain searches by CD-Search at National Center for Biotechnology Information (Marchler-Bauer and Bryant 2004). Function was inferred from a combination of information gained from the conserved domain searches, FlyBase classification, Gene Ontology database classification, and literature searches.

Results

Coding Sequences in the cDNA Library

Of the 383 EST library clone sequences, 244 matched coding sequences (CDSs) in the *D. simulans* genome repre-

sented 44 unique CDSs. The remaining 139 sequences were short with typically less than 20 base pairs matched against any *D. melanogaster* or *D. simulans* CDSs, and had E values greater than 0.01 (accepted sequences had an average E value of 4.5×10^{-6}). These sequences were excluded from further analysis because they did not meet the criteria described in "Materials and Methods."

Orthologs and Functional Categories

Sequence similarity was used to identify orthologs in seven *Drosophila* genomes: *D. simulans*, *D. melanogaster*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, and *D. pseudoobscura*. Of the 44 genes identified from the library, 29 had identifiable orthologous genes in all seven species (31 genes had identifiable orthologs in all six species when excluding *D. pseudoobscura*). For the remaining 15 genes orthologs were found in some, but not all of the seven species. For two of 44 genes (dsim_GLEANR_6594 and dsim_GLEANR_15604) there were no orthologous genes shared by *D. simulans* and *D. melanogaster*. Orthologs to these genes were found in other *Drosophila* species (dsim_15604 was found in *D. erecta* and *D. yakuba*; dsim_6594 was found in *D. sechellia*, *D. erecta*, and *D. yakuba*), but were not used for molecular evolutionary analysis. The method used to identify orthologs was comprehensive and it sometimes revealed annotation errors. For example, two *D. simulans* genes (dsim_GLEANR_16297 and 17130) appeared to be an incorrect annotation of two exons from a larger gene based on the gene structure of the *D. melanogaster* ortholog (CG32702). Therefore, these two exons were combined into one gene for *D. simulans* as well as in *D. erecta* and *D. ananassae* (dere_GLEANR_3759 and 3760, and dana_20818 and 20819).

Gene function was investigated for all genes having orthologs present in both *D. simulans* and *D. melanogaster* (42 genes in total; Table 1, Supporting Table 1). The most likely function was determined by the Gene Ontology database, conserved domains, and relevant literature. Active serine proteases are indicated by the presence of three residues (Ser195, Asp102, and His57) termed the catalytic triad. Identification of residues of the catalytic triad was done using the SMART (Schultz *et al.* 1998; Letunic *et al.* 2006). Of the 42 genes, 11 are putative serine proteases 10 of which have the catalytic triad of amino acids. The substrate specificity of serine proteases is due to residues surrounding Ser195 (Perona and Craik 1995; Hedstrom 2002), these areas were identified in the proteases of this study. Positive selection was predicted to be operating in regions adjacent to the substrate specificity sites (Fig. 1; Supporting Figure S1). Expression Analysis Systematic Explorer (EASE) integrated into the DAVID bioinformatics database (<http://david.abcc.ncifcrf.gov/home.jsp>) indicated that proteases were significantly (P -value 4.5×10^{-9}) overrepresented in the spermathecae (26%) when compared to the percentage of such genes in the *D. melanogaster* genome (5%) (Ross *et al.* 2003). We

Table 1. Functional annotation of 42 genes enriched for expression in the spermatheca

Function ¹	Number ²	SP ³	TM ⁴
Serine protease	11	11	
Cell communication	3		2
Peptidase	3	2	2
Translation	2		
Actin formation/biosynthesis	2		
Amino acid transport	1	1	1
Antimicrobial	1	1	
Apoptosis	1		1
Cation transport	1		1
Dehydrogenase	1	1	
Helicase	1		
Juvenile hormone catabolism	1	1	1
Nerve signaling	1		1
Phospholipid metabolism	1	1	1
Secondary metabolism	1		
Protein-protein interaction	1		
Sugar metabolism	1	1	
Unknown	9	4	3

¹Predicted function of encoded proteins.²Number of genes.³Number of genes predicted to encode proteins that have secretion signal peptides.⁴Number of genes predicted to encode proteins with transmembrane regions.

do not assume that an exhaustive sampling of the spermathecae library was performed; nevertheless, there is no reason to assume a bias in the choice of clones for sequencing. The relative proportions of genes in the categories listed are expected to be similar to the actual proportions. One of the genes (dsim_GLEANR_6594) discovered

in the library, but having no *D. melanogaster* ortholog, is also predicted to be a serine protease.

Evolutionary Analyses

Nonsynonymous and synonymous substitution rates were determined for each of the 42 genes. One analysis was a pairwise comparison between *D. simulans* and *D. melanogaster*. The rates of synonymous and nonsynonymous substitutions were calculated by a maximum-likelihood method using PAML (Yang 1997, 2007). The average d_N/d_S ratio from the pairwise comparisons between the *D. simulans* and *D. melanogaster* sequences is 0.269 ± 0.2932 , with an average d_N of 0.032 and an average d_S of 0.119. In this comparison, 10 of the 42 genes (Fig. 2) have d_N/d_S higher than the 0.5 threshold adopted by Swanson *et al.* (2004). Results of the branch-model PAML analysis supported the *D. melanogaster*/*D. simulans*/*D. sechellia* lines subject to positive selection for five of the 10 genes identified above (Supporting Table S2). Only one gene (CG15098) constantly showed positive selection both in the *D. melanogaster* and *D. sechellia* lineages regardless of the placement of *D. yakuba* and *D. erecta*. On the other hand, six genes (including five not identified by pairwise comparisons) showed possible positive selection in the *D. simulans*/*D. sechellia* lines.

Another analysis compared the fit of the data to different models of codon evolution (Yang and Nielsen 2000). The "site-model" analysis in PAML was used to explore heterogeneity in d_N/d_S along the gene, and to test for positive Darwinian selection (Table 2, Supporting Table 1). These comparisons were restricted to the 40 genes for which sequences were available from at least four species. The first comparison examined the fit of data to the

CG17234	sim	YRR TACH GD <u>SGG</u> PLVVKQLVGVVSWG RKGC [*] QSSTFFV SVPY FREW
	mel	YGR TACH GD <u>SGG</u> PLVVKQLVGVVSWG RKGC VSSAFFV SVPY FREW
	sec	YRR TACH GD <u>SGG</u> PLVVKQLVGVVSWG RKGC QSSTFFV SVPY FREW
	yak	YGK TVCE GD <u>SGG</u> PLVVKQLVGVVSWG KRDC SSRAFFA SVPY YREW
	ere	TGR AAAC RGD <u>SGG</u> PLVANKQLVGVVSGG SEYCE KSSYY SVPY FHEW
	pse	SHK STLF ED <u>SGG</u> PLTVNKQLVGVVCGGR YG --SPIMY SVIY HKDW
Ser12	sim	L GRD ACRGD <u>SGG</u> PLVSGGQLVGVIVSYGIGCANPFFPGV YANVA [*] VLK
	mel	LLK DSC HGD <u>SGG</u> PLVSGGQLVGVIVSYGIGCANPFFPGV YANVA ELK
	yak	LLK DSC QGD <u>SGG</u> PLVSGGQLVGVIVSHGMLCAIPFYPGV YTNVA VLK
	ere	L GKD SCQGD <u>SGG</u> PLVSGGQLVGVIVSFGYGCANPFFPGV YANVA HLK
CG31681	sim	HRW DSC EGD <u>SGG</u> PLIEITTGG- [*] HELVGIVSWGEGCGTN LGVY EDIA
	mel	QRW DTC QGD <u>SGG</u> PLIETTKGGHRQLIGM VS WGDGCGTN PGVY EDIA
	yak	HRG DTCE GD <u>SGG</u> PLVDTKN---KKLVGIVSWGVGCGIN PGVY ADVA
	ere	PGR SACR GD <u>SGG</u> PLVEMET---RKLIGIVSWGFRCGTA PGVY ADVA

Figure 1. Serine proteases identified by PAML as having positively selected codons flanking the serine active site (bold and underlined). Amino acid residues in bold are integral for serine protease substrate specificity. * indicates amino acid residues that were significant for positive selection using PAML. See Supporting Table S3 for a complete list the serine protease active site residues of the serine proteases discovered in the present study.

one-ratio model (M0) against the model that classifies sites into three classes (M3). For 37 of the 40 genes, the fit of data to M3 was significantly better than M0, indicating heterogeneity of evolutionary rates along the gene for a high percentage of the genes analyzed. Direct tests of positive selection were also performed. Briefly, two likelihood-ratio tests (LRTs) were used to compare null models that do not allow $d_N/d_S > 1$, M1a (nearly neutral) and M7 (beta), with alternative models that allow a class of sites to have $d_N/d_S > 1$, M2a (positive selection) and M8 (beta and omega) (Yang and Nielsen 2002; Yang and Swanson 2002). In the first test, the null model (M1a) assumes two site classes, the first with d_N/d_S close to 0, and the second with $d_N/d_S = 1$; this is compared with the alternative model (M2a) that adds a class of sites with $d_N/d_S > 1$. The second test uses M7 as the null model, where d_N/d_S estimates are drawn from a beta distribution with $0 \leq d_N/d_S \leq 1$, with the alternative model M8, which adds a class of sites with $d_N/d_S > 1$. If the LRTs were significant, positive selection was inferred (Yang and Nielsen 2002; Yang and Swanson 2002). Comparisons of M7 to M8 and M1 to M2 provided evidence for positive selection in 17 of 40 genes. Genes having elevated d_N/d_S from the pairwise comparison, and/or support for positive selection from PAML models using trees excluding *D. pseudoobscura*, are listed in Table 2. All values are listed in Supporting Table S1, including the results of analysis including *D. pseudoobscura*.

Results of the branch model analysis show seven genes in the *D. simulans*/*D. sechellia* lines subject to positive selection. Only one gene (CG15098) constantly showed positive selection in all three topologies in the *D. melanogaster* lineage. All data from PAML branch model analysis are reported in Supporting Table S2.

To obtain extended taxonomic insight into the evolution of spermathecal genes, the presence or absence of homologous genes was examined comparing *D. melanogaster* to 11 sequenced genomes (*D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis*, *D. virilis*, and *D. grimshawi*) (Supporting Table S1). A number of genes, including predicted proteases, a peptidase, an actin biosynthesis gene, Drosomycin, and a gene of unknown function, were not detectable in taxa distant from the *melanogaster* subgroup (*D. mojavensis*, *D. virilis*, and *D. grimshawi*). A high percentage of the genes with undetectable orthologs in more distantly related species encode serine proteases (see Discussion).

Possible species-specific duplications were found in *D. erecta*, *D. ananassae*, and *D. pseudoobscura*. Duplicated copies in *D. erecta* (dere_GLEANR_9251, dere_GLEANR_13114, dere_GLEANR_16834) and in *D. ananassae* (dana_GLEANR_20091 and dana_GLEANR_20093) share 80 and 90% similarities, respectively, against their corresponding *D. simulans* genes. A pair of *D. ananassae* genes (dana_GLEANR_9014 and dana_GLEANR_10165) was almost

identical (only one nucleotide difference) to a pair of *D. pseudoobscura* genes (dpse_GLEANR_6308 and dpse_GLEANR_6306). Further investigation would be needed to determine if these apparent duplications are due to artifacts such as assembly mistakes.

Secretion Signal Sequence and Transmembrane Region Prediction

Of the 42 genes examined, 23 (55%) are predicted to have signal peptides and 13 (31%) are predicted to have transmembrane regions (Table 1). Three of the genes predicted to have transmembrane regions and 11 of the genes predicted to have signal peptides show evidence of positive selection (Supporting Table S1). All of the proteases have predicted secretion signal sequences.

Discussion

Rapidly evolving reproductive proteins are candidates to play an important role in sexual selection and speciation. To identify candidate genes that could play a role in these evolutionary processes, the molecular evolution of genes expressed in the spermatheca was analyzed and likely gene function was characterized. A high proportion of spermatheca genes are predicted to encode serine proteases many of which evolve rapidly, and all have secretion signals. Serine proteases expressed in the spermatheca are prime candidates to participate in evolutionarily dynamic interactions with male seminal products. Overall, a high percentage of the genes exhibit the molecular signal of positive selection. Insight into the function of the spermatheca was obtained from the identity of genes expressed in this SSO.

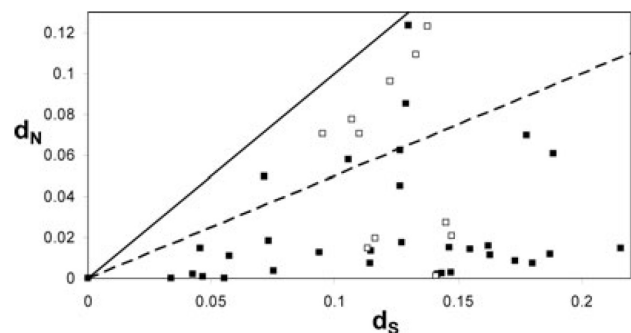


Figure 2. The number of nonsynonymous substitutions per site (d_N) plotted against the number of synonymous substitutions per site (d_S) for the *D. simulans* spermathecae EST library. The solid line represents d_N/d_S of 1.0, the historical threshold for positive selection. The dashed line represents d_N/d_S of 0.5, the threshold for the identification of candidate gene for positive selection. All squares on the graph represent genes found in the spermathecae EST library and open squares correspond to the 11 serine proteases.

Table 2. Genes with elevated pairwise d_N/d_S (>0.5) and genes identified as having regions that provide evidence for evolution by positive selection (PAML analysis)

Genes	Function ¹	d_N/d_S ²	Species ³	M0 vs. M3		M1 vs. M2		M7 vs. M8		pos. sel ¹⁰
				P_S ⁴	d_N/d_S ⁵	P_S ⁶	d_N/d_S ⁷	P_S ⁸	d_N/d_S ⁹	
CG8331	cell comm.	0.19	M,S,Sc,Y,E,A	0.01	30.56 ¹⁴	0.08	1.00	0.01	30.61 ¹²	1
CG10650	peptidase	0.55	M,S,Sc,Y,A	0.11	2.94 ¹⁴	0.03	4.93	0.06	3.67 ¹²	
CG32702	protein int.	0.14	M,S,Sc,Y,E	0.02	2.55 ¹⁴	0.00	6.09	0.01	3.58 ¹³	
CG3066	ser. protease	0.17	M,S,Sc,Y,E,A	0.03	2.56 ¹⁴	0.04	1.00	0.02	2.83 ¹²	
ηTry	ser. protease	0.14	M,S,Sc,Y,E,A	0.03	3.02 ¹⁴	0.01	3.74	0.03	3.26 ¹²	3
Ser12	ser. protease	0.64	M,S,Y,E	0.09	6.27 ¹⁴	0.09	6.25 ¹⁴	0.09	6.25 ¹⁴	9
CG17012	ser. protease	0.89	M,S,Sc,A	0.07	6.08 ¹⁴	0.05	8.21 ¹⁴	0.06	6.96 ¹⁴	
CG17234	ser. protease	0.74	M,S,Sc,Y,E	0.05	7.23 ¹⁴	0.09	5.25 ¹⁴	0.10	4.86 ¹⁴	
CG17239	ser. protease	0.73	M,S,Sc,Y,E,A	0.16	2.71 ¹⁴	0.09	3.41 ¹⁴	0.13	2.94 ¹⁴	6
CG18125	ser. protease	0.79	N/A							
CG31681	ser. protease	0.82	M,S,Y,E	0.09	5.57 ¹⁴	0.08	6.05 ¹⁴	0.09	5.84 ¹⁴	6
Treh	sugar metab.	0.12	M,S,Sc,Y,E,A	0.04	3.36 ¹⁴	0.02	5.51 ¹⁴	0.03	3.75 ¹⁴	5
Ef1α48D	translation	0.02	M,S,Sc,Y,E,A	0.01	2.11 ¹⁴	0.01	2.11	0.01	2.11 ¹²	4
Qm	translation	0.02	M,S,Sc,Y,E,A	0.005	3.25 ¹⁴	0.01	3.25	0.01	3.25 ¹³	1
CG2233	unknown	0.95	M,S,Sc,Y,E	0.02	33.31 ¹⁴	0.18	4.16 ¹⁴	0.17	4.37 ¹⁴	
CG11137	unknown	0.1	M,S,Sc,Y,E,A	0.01	1.80 ¹²	0.00	25.23	0.01	1.80 ¹²	1
CG15098	unknown	0.69	M,S,Sc,Y,E,A	0.09	2.77 ¹⁴	0.04	3.66	0.07	3.00 ¹²	1
CG30197	unknown	0.05	M,S,Sc,Y,E	0.02	4.37 ¹³	0.02	4.37	0.02	4.37 ¹²	
CG31686	unknown	0.66	N/A							

¹Predicted protein function.² d_N/d_S : pairwise comparison of *D. melanogaster* and *D. simulans* sequences, estimated assuming no rate heterogeneity.³Species: refers to the species of *Drosophila* from which sequences were obtained for PAML analysis M, *melanogaster*; S, *simulans*; Sc, *sechellia*; Y, *yakuba*; E, *erecta*; A, *ananassae*.

N/A indicates that PAML analysis was not done due to too few species. The following statistics are all derived from PAML analysis.

⁴ P_S : the proportion of sites estimated to belong to the class that has the highest d_N/d_S in M3.⁵ d_N/d_S : for the highest class in M3.⁶ P_S : the proportion of sites estimated to belong to the class that has $d_N/d_S > 1$ in M2.⁷ d_N/d_S : the estimate for the class with the ratio > 1 in M2.⁸ P_S : the proportion of sites estimated to belong to the class that has $d_N/d_S > 1$ in M8.⁹ d_N/d_S : the estimate for the class with the ratio > 1 in M8.¹⁰pos.sel.: the number of codons significantly ($P < 0.05$) recognized by PAML as being positively selected;¹² $P < .05$; ¹³ $P < .01$; ¹⁴ $P < .001$.

In the present study, 44 unique *D. simulans* genes were identified in the hybrid-selected cDNA library. Orthologs were discovered in *D. melanogaster* for 42 of the 44 genes. The small number of genes found in this study is quite similar to the number of genes found in comparable studies in *Drosophila* and other species. (DiBenedetto *et al.* 1987; Monsma and Wolfner 1988; Wolfner *et al.* 1997; Swanson *et al.* 2001a; Andres *et al.* 2006; Davies and Chapman 2006).

It is informative to compare the evolution of the 42 spermatheca genes identified in this study with relevant previous studies. Based on the pairwise comparison between *D. simulans* and *D. melanogaster*, 24% of the spermatheca genes have an overall $d_N/d_S > 0.5$ (Fig. 1). Two especially relevant previous studies (Swanson *et al.* 2001a, 2004) have made pairwise comparisons of *D. simulans* and *D. melanogaster*. The incidence of genes with $d_N/d_S > 0.5$ in

the spermatheca (present study) is at least twice as high as observed in the female reproductive tract of *D. melanogaster*, minus ovaries, in which 6% of the genes had a $d_N/d_S > 0.5$ (Swanson *et al.* 2004) and at least as high as that observed for male accessory gland genes among which 19% of genes had a $d_N/d_S > 0.5$ (Swanson *et al.* 2001a). The number of genes in the present study that overlapped with the most similar study (Swanson *et al.* 2004) was only five, even though their study included spermathecae in the mix of tissue investigated. The small overlap between Swanson (2004) and the present study could be attributed to the small proportion of tissue mass contributed by the spermathecae to the lower reproductive tract. Genes expressed in the spermathecae might be represented in such low levels compared to genes from larger tissues that they were undetected in the lower reproductive tract cDNA library. Alternately, a number of technical

differences could be responsible for the small overlap in genes between these two studies, including the processes used to screen and select clones or the use of females of different ages. Of the 10 spermathecal genes with elevated d_N/d_S (> 0.5), the most rapidly evolving was a gene of unknown function ($d_N/d_S = 0.95$), followed by five serine proteases ($d_N/d_S = 0.72 - 0.89$), and two more genes of unknown function ($d_N/d_S = 0.64 - 0.66$). Acp male genes are notable for their rapid rates of evolution, and it appears that female spermatheca genes are similarly evolutionarily dynamic.

The analysis of the pattern of molecular evolution among a larger set of related species is also informative. Molecular evolution analyses showed that 17 of 40 (42.5 %) spermatheca genes contain at least one region that conforms to a model of positive selection (Table 2). It has been suggested that tests, such as PAML, which involved the fitting of a distribution of substitution rates across sites as a method for inferring individually evolving sites may be prone to type I (Suzuki and Nei 2004) or type II (Kosakovsy and Frost 2005) errors. This is especially a problem when the test species are highly similar, or if too few species are used (Anisimova *et al.* 2001). The current analysis uses species of *Drosophila*, which are expected to be sufficiently divergent to minimize the amount of type II error. The simulation study performed by Kosakovsy and Frost (2005) also showed that with their eight-sequence datasets, PAML (M8 model) performed as well as all other approaches. Analysis performed in the current study using five, six, or seven species showed consistent results (Supporting Table S1). Categories of genes showing evidence for positive selection include serine proteases, cell communication, translation, sugar metabolism, peptidase activity, protein-protein interaction and genes of unknown function (Table 2). The proportion of positively selected genes can be compared to molecular evolution of *Drosophila* seminal fluid proteins using the *melanogaster* species subgroup (Haerty *et al.* 2007). Twenty-five seminal fluid genes had orthologs in all of the *melanogaster* subgroup species and four of these genes (16%) exhibited positive selection by the criteria of acceptance of M8 over M7. By the same criteria, of 679 genes expressed in the reproductive tract of *D. melanogaster*, and of 9921 nonsex/reproduction-related genes, 6.2% and 6.0%, respectively, were consistent with the hypothesis of positive selection by acceptance of model 8 (Haerty *et al.* 2007). It is important to note that direct comparisons made between studies are tempered by the technical differences of the individual studies. For example, a hybrid selection study such as the present study is expected to be based on a more restricted set of genes than other approaches. Nevertheless, the incidence of directional selection among spermathecal genes is striking.

In the present study, serine proteases are the predominant category of genes lost from *D. melanogaster* and *D. simulans* as a function of evolutionary distance. Among spermatheca proteases, four have no detectable orthologs

in species belonging to the *melanogaster* subgroup and five protease genes have no detectable ortholog in the *obscura* subgroup. At the level of differentiation between *D. melanogaster*/*D. simulans* and the *repleta* group, seven protease genes have no orthologs. Between the *melanogaster* subgroup and a Hawaiian *Drosophila*, 10 protease genes have no orthologs. Rapid evolution of protease genes in the *melanogaster* subgroup (and related species) continues until most spermathecal protease genes are lost, or no longer recognizable as an ortholog, in more distantly related taxa. In other studies using *Drosophila* species, reproductive system proteases show evidence of accelerated and positive evolution (Kern *et al.* 2004; Swanson *et al.* 2004; Panhuis and Swanson 2006; Kelleher *et al.* 2007; Lawniczak and Begun 2007; Wong *et al.* 2008) the proportion of such genes in the spermatheca is the highest recorded considering these relevant studies.

All of the spermatheca serine proteases have secretion signals (Table 1) and are possibly secreted into the lumen of this SSO. Potential roles for male and female proteases are discussed in Ravi-Ram and Wolfner (2007). Spermathecal proteases may be involved in interactions with male reproductive proteins, or play roles functionally analogous to male reproductive proteins. Previous studies have described at least two *Drosophila* male proteins that are transferred to females and undergo cleavage within the female reproductive tract, perhaps as a mechanism to control activity levels of the proteins (Monsma *et al.* 1990; Bertram *et al.* 1996; Ravi-Ram and Wolfner 2007). Female proteases might act to control the viscosity of the internal milieu of the lumen of the spermatheca analogous to the semen coagulation role played by the primate prostate-specific antigen (PSA) in males (Malm *et al.* 2000). PSA is a serine protease and its role in humans suggests an analogous function for spermathecal proteases in *Drosophila*.

The evolutionary importance of the interactions that occur between females and male ejaculate are being increasingly recognized. As an exciting possibility, male-derived protease inhibitors might inhibit female proteases secreted into the lumen spermatheca in a specific male-female (ejaculate-female) molecular interaction. Seven protease inhibitors have been reported among Acps of *D. melanogaster*. Acp 62F, which is able to transverse the female reproductive tract and enter the hemolymph, is toxic upon ectopic expression; this Acp is present in the spermatheca after mating (Lung *et al.* 2002). An Acp protein that plays a key role in sperm storage (Acp36DE) is found in the spermatheca after mating and it is rapidly evolving. Moreover a protease Acp associated with regulation of sperm use is also evolving rapidly (Wong *et al.* 2008) and it also is a candidate for a coevolutionary interaction with spermatheca proteases based on direct interaction.

Four of the protease genes identified in the present study are found in a cluster on the chromosomal arm 2L. These genes exhibit approximately 30% sequence similarity to each other in *D. melanogaster* and each gene is ap-

proximately 90% similar to its ortholog in *D. simulans*. They have no introns and they encode proteins with the canonical serine protease catalytic triad of amino acids. The cluster of proteases has been found to be transcriptionally activated by mating (Lawniczak and Begun 2007). These proteases, and several others, are rapidly evolving between populations of *D. melanogaster* and diverging between *D. melanogaster* and *D. simulans*. (Lawniczak and Begun 2007). This rapid divergence can further be exemplified by the two spermatheca genes found in *simulans* without a *melanogaster* ortholog (see Results). One of these genes, *dsim_GLEANR_6594*, is found in the middle of the four clustered proteases in the *D. simulans* genome, and is predicted to be a serine protease based on conserved domains. A large corresponding portion of this region is missing from the *D. melanogaster* genome, which provides an explanation for the lack of ortholog found in *D. melanogaster* and yields a picture of rapid change between the two genomes.

Five of the proteases found in the present study (CG18125, and the cluster on chromosome II) have been foci for previous molecular population genetic and molecular evolution studies. These studies showed that the sites of molecular changes in these proteases were associated with the active site, suggesting the evolution of functional changes related to catalysis (Panhuis and Swanson 2006; Lawniczak and Begun 2007). Three sites surrounding Ser195, one of the three identified positions of the serine protease catalytic triad, have been identified as responsible for substrate specificity (Perona and Craik 1995; Hedstrom 2002) were examined in the serine proteases of the present study. A variety of changes were seen in and around these regions, suggesting that the changes affected catalysis and substrate specificity (Fig. 1; Supporting Figure S1). CG18125 was also found to be significantly induced by mating (McGraw *et al.* 2004). The expression of CG18125 in mated females was over twice that of virgin females.

One class of spermathecal proteins identified in this study contains at least one protein-protein interaction motif called a CUB domain. CUB domains, which consist of approximately 110 amino acids with four positionally conserved cysteines, (Bork and Beckman 1993) play a variety of roles including interaction with sperm in both vertebrate and invertebrate taxa (Kamei and Glabe 2003; Haley and Wessel 2004). CUB domains bind other proteins with high specificity (Song *et al.* 2006) and tend to exist as a cluster of multiple repeats along the length of a single gene. A gene identified in the present study, CG32702, contains approximately 20 CUB domains in one region of the protein, along with a repeat of five EGF-CA-like domains at the C-terminal end. A second gene (CG30371) encodes a trypsin-like serine protease domain and a motif that is 67% similar to a CUB domain. CG32702 (the gene with many CUB domains) exhibits evolutionary stasis in much of the gene, but relatively

rapid evolution in regions of the gene. Having multiple CUB domains potentially allows for a relaxation of selective constraints. Changes could be tolerated in a subset of the domains because the original specificity may be retained by the remaining (unchanged) domains. A general argument about redundancy and relaxation of selective constraints when repeated motifs are present in a protein has been made by Metz and Palumbi (1996) and is used to interpret the evolution of VERL domains in reproductive proteins (Swanson and Vacquier 1998). There is evidence for positive selection in regions of the CUB protein even though the protein is sufficiently conserved to be found in all 12 sequenced *Drosophila* genomes.

Other genes-encoding proteins with potentially important roles associated with sperm storage and maintenance were identified. Trehalase activity (sugar metabolism gene in Table 1) could play a role in sperm nutrition. The *Drosophila* trehalase RNA encodes a predicted secretion signal and thus its protein could be active in the lumen of the spermatheca. In honey bees the spermathecal fluid contains sugars including glucose, trehalose, and fructose, as well as a high level of trehalase activity (Alumot *et al.* 1969). A gene encoding an antifungal defense peptide (Drs) also was identified in the present study. This gene is not spermatheca specific, it is constitutively expressed in both types of SSOs of *D. melanogaster* (Ferrandon *et al.* 1998). The SSOs are apparently the only site of constitutive expression whereas the gene is expressed in many locations after induction with a pathogen (Ferrandon *et al.* 1998). Juvenile hormone epoxide hydrolase 3 (JHEH3) is an example of a gene that could play an interesting role in evolution. This enzyme catabolizes juvenile hormone (JH) to an inactive metabolite. Its protein product is predicted to have a secretion signal and six transmembrane domains suggesting it could be a receptor. It is possible that JHEH3 could be acting to control JH levels in the spermatheca and as a systemic hormone regulator if it is secreted into the hemolymph. In *D. melanogaster*, Acp70 is transferred to females at the time of mating, which stimulates JH synthesis (Peng *et al.* 2005). An enzyme that produces a precursor of JH is elevated in the lower female reproductive tract after mating (Mack *et al.* 2006). The presence of enzyme activity that degrades juvenile hormone (JHEH3) in the long-term SSO is intriguing because it might oppose the male effect of stimulating the synthesis of JH. Genes with unknown function in *Drosophila* or other species (Table 1) might be quite interesting in terms of having spermatheca-specific roles because functions for such genes have not been identified in other tissues or taxa. Four of these genes have transmembrane domains that could be receptors having spermatheca-specific function. These receptors could potentially interact with male accessory gland proteins or other proteins found on sperm. Identification of such receptors would be important for understanding the evolution of Acp's and how they function in females.

Conclusions

This study has produced insight into the evolution and function of genes enriched for expression in *Drosophila* spermathecae. We find that genes expressed in the spermatheca evolve as rapidly as genes in the male accessory gland. Importantly, the proportion of genes with the overall signature of positive selection is higher than that of Acp genes that are a paradigm for rapid evolution. Rapidly evolving spermatheca proteins of established and novel function could participate in female reproductive molecule-ejaculate interactions that are increasingly recognized as evolutionarily important (Ravi-Ram and Wolfner 2007).

Acknowledgments

We thank M. Wolfner, A. Wong, W. Wagner, M. Noor, C. Aquadro, and A. Clark for comments on the manuscript and other contributions to the research. This research was supported by a National Science Foundation grant (DEB-ESP0346476).

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The following supporting information is available for this article:

Figure S1. Amino acid sites surrounding the serine active site (bold and underlined) of the catalytic triad for the serine proteases discovered in the spermathecae.

Table S1. Gene identification numbers for *D. simulans* and *D. melanogaster*. *D. melanogaster* CG and FB gene identification numbers; Function of predicted proteins; Secretion Signal prediction; Transmembrane region prediction; Pairwise d_N/d_S ; PAML data; Presence of orthologs in 12 genomes

Table S2. Gene identification numbers for *D. simulans* and *D. melanogaster*; Pairwise d_N/d_S ; PAML branch model data.

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These materials are also presented on the following pages.

Supplementary Figure 3. Amino acid sites surrounding the serine active site (bold and underlined) of the catalytic triad for the serine proteases discovered in the spermathecae. Residues in bold represent the sites involved in substrate specificity. Residues in italics represent sites predicted to be under positive selection by PAML analysis.

CG3066	sim	FYRD <u>SCD</u> GD <u>SGG</u> PLMRRGFNQAWYQEGV <u>VS</u> <u>FG</u> NRCGLE <u>GW</u> PGVYTR
	mel	FYRD <u>SCD</u> GD <u>SGG</u> PLMRRGFDQAWYQEGV <u>VS</u> <u>FG</u> NRCGLE <u>GW</u> PGVYTR
	sec	FYRD <u>SCD</u> GD <u>SGG</u> PLMRRGFNQAWYQEGV <u>VS</u> <u>FG</u> NRCGLE <u>GW</u> PGVYTR
	yak	FYRD <u>SCD</u> GD <u>SGG</u> PLMRRGFDQAWYQEGV <u>VS</u> <u>FG</u> NRCGLE <u>GW</u> PGVYTR
	ere	FYRD <u>SCD</u> GD <u>SGG</u> PLMRRGFDQAWYQEGV <u>VS</u> <u>FG</u> NRCGLE <u>GW</u> PGVYTR
	ana	FYKD <u>SCD</u> GD <u>SGG</u> PLMREGFNHAWYQEGV <u>VS</u> <u>FG</u> NRCGLE <u>GW</u> PGVYTR
	pse	FYRD <u>SCD</u> GD <u>SGG</u> PLMRRGFQAWFQEGV <u>VS</u> <u>FG</u> NRCGLE <u>GW</u> PGVYTS
CG10469	sim	KKGL <u>PCR</u> GD <u>SGG</u> PMVLDDGTRTLVGIVSH <u>G</u> FDGECKLK <u>LPD</u> ISTRV
	mel	KKGL <u>PCR</u> GD <u>SGG</u> PMVLDDGSRTLVGIVSH <u>G</u> FDGECKLK <u>LPD</u> VSTRV
	sec	KKGL <u>PCR</u> GD <u>SGG</u> PMVLDDGTRTLVGIVSH <u>G</u> FDGECKLK <u>LPD</u> ISTRV
	yak	KKGL <u>PCR</u> GD <u>SGG</u> PMVLDDGSKTLVGIVSH <u>G</u> FDGECKLK <u>VPD</u> ISTRV
	ere	KEGL <u>PCR</u> GD <u>SGG</u> PMVLDDSTRTLVGIVSH <u>G</u> FDGECKLK <u>LPD</u> ISTRV
	ana	KYGM <u>P</u> CQGD <u>SGS</u> PMVLADGSKTLVGIVSH <u>G</u> LDPA CKRK <u>VPD</u> ISM RV
	pse	RAGL <u>PCR</u> GD <u>SGG</u> PLVLDDGSRTLVGIVSH <u>G</u> YDAK CQVK <u>LPD</u> VLTRV
etaTry	sim	GGK <u>DAC</u> QGD <u>SGG</u> PLVVANKLAGIVSWGEG <u>C</u> ARPNYPGV <u>YAN</u> VAYFK
	mel	GGK <u>DAC</u> QGD <u>SGG</u> PLVVANKLAGIVSWGEG <u>C</u> ARPNYPGV <u>YAN</u> VAYYK
	sec	GGK <u>DAC</u> QGD <u>SGG</u> PLVVANKLAGIVSWGEG <u>C</u> ARPNYPGV <u>YAN</u> VAYFK
	yak	GGK <u>DAC</u> QGD <u>SGG</u> PLVVANKLSGIVSWGEG <u>CA</u> QPNYPGV <u>YAN</u> VAYFK
	ere	GGK <u>DAC</u> QGD <u>SGG</u> PLVVANKLAGIVSWGEG <u>C</u> ARPNYPGV <u>YAN</u> VAYFK
	ana	GGK <u>DAC</u> QGD <u>SGG</u> PLVGNKLAGIVSWGEG <u>C</u> ARPNYPGV <u>YAN</u> VAYFK
	pse	GGK <u>DAC</u> QGD <u>SGG</u> PLVVNNQLVGIVSWGEG <u>C</u> ARPNTPGV <u>YAN</u> VAHFK
CG17012	sim	LGK <u>AGC</u> GD <u>SGG</u> PLVFNDQLVGITSRK <u>GNLL</u> CLASSLYASVAYYRN
	mel	MKG <u>GCY</u> GD <u>SGG</u> PLVFNGQLVGITSRT <u>GNIV</u> CLGSSLYASVARYRN
	sec	IGK <u>GGC</u> GD <u>SGG</u> PLVFNDQLVGITSRK <u>GNLL</u> CLSSTLYASVAYYRT
	ana	VGH <u>AHCF</u> GD <u>SGG</u> PLVVEKTLVGISSRT <u>GNAF</u> CLSPGYVSVAKFRP
CG17234	sim	YRR <u>TACH</u> GD <u>SGG</u> PLVVNKQLVGVVSWGR <u>KGC</u> Q SSTFFV <u>SV</u> PYFREW
	mel	YGR <u>TACH</u> GD <u>SGG</u> PLVVNKQLVGVVSWGR <u>KGC</u> V SSAFFV <u>SV</u> PYFREW
	sec	YRR <u>TACH</u> GD <u>SGG</u> PLVVNKQLVGVVSWGR <u>KGC</u> Q SSTFFV <u>SV</u> PYFREW
	yak	YGK <u>TVCE</u> GD <u>SGG</u> PLVVNKQLVGVVSWGK <u>RDC</u> S SRAFFAS <u>SV</u> PYYREW
	ere	TGR <u>AACR</u> GD <u>SGG</u> PLVANKQLVGVVSGGS <u>EYCE</u> KSSYYSS <u>SV</u> PYFHEW
	pse	SHK <u>STLF</u> ED <u>SGG</u> PLTVNKQLVGVVCGGR <u>YG</u> - - SPIMYSS <u>SV</u> IYHKDW
CG17239	sim	PGK <u>DACS</u> GD <u>SGG</u> PLVSGDKLVGIVSFGK <u>ECA</u> HSKFPGV <u>YAN</u> VAELK
	mel	PGK <u>DACS</u> GD <u>SGG</u> PLVSGNKLVGIVSFGK <u>ECA</u> HPEYPGV <u>YAN</u> VAELK
	sec	PGK <u>DACS</u> GD <u>SGG</u> PLVSGNKLVGIVSFGK <u>ECA</u> HSKFPGV <u>YAN</u> VAELK
	yak	PGK <u>DACS</u> GD <u>SGG</u> PLVSDGKLVGIVSFGA <u>KCAL</u> PKYPGV <u>YAN</u> VAVLK
	ere	PGK <u>DACT</u> GD <u>SGG</u> PLVSGGQLVGIVSFGA <u>ECA</u> LPQFPGV <u>YAN</u> VAELK
	ana	PGK <u>DACQ</u> GD <u>SGG</u> PLVSGGKLVGIVSFG <u>NGCA</u> HPRYPGV <u>YSN</u> VAVLR

Supplementary Figure 3 (continued). Amino acid sites surrounding the serine active site (bold and underlined) of the catalytic triad for the serine proteases discovered in the spermathecae. Residues in bold represent the sites involved in substrate specificity. Residues in italics represent sites predicted to be under positive selection by PAML analysis.

Ser12	sim	LGR DAC <u>R</u> GD <u>S</u> GGPLVSGGQLVGIVSYG IG <u>C</u> ANPFFPGV YAN VAVLK
	mel	LLK D <u>S</u> C HGD <u>S</u> GGPLVSGGQLVGIVSYG IG <u>C</u> ANPFFPGV YAN VAELK
	yak	LLK D <u>S</u> C QGD <u>S</u> GGPLVSGGQLVGIVSHGML CA IPFYPGV YTN VAVLK
	ere	LK D <u>S</u> C QGD <u>S</u> GGPLVSGGQLVGIVSFGY G <u>C</u> ANPFFPGV YAN V AH LK
CG18125	sim	YGR TAC <u>N</u> GD <u>S</u> GGPLVVGQQLVGVVSGGSE ED CTYSALYTS VP <u>P</u> YFREW
	mel	FGR AAC <u>K</u> GD <u>S</u> GGPLVFDQQLVGVVSGGT KD CTYSSYTS VP <u>P</u> YFREW
	sec	YGR TAC <u>N</u> GD <u>S</u> GGPLVVGQQLVGVVSGGSE ED CTYSALYTS VP <u>P</u> YFREW
CG30371	sim	TGR D <u>S</u> C QFD <u>S</u> GGPVILRKSQFLVGIISY GK SCAESQY PM <u>G</u> VNTRI
	mel	TGR D <u>S</u> C QFD <u>S</u> GGPVILRKSQFLVGIISY GK SCAESQY PM <u>G</u> VNTRI
	sec	TGR D <u>S</u> C QFD <u>S</u> GGPVILRKSQFLVGIISY GK SCAESQY PM <u>G</u> VNTRI
	yak	TGR D <u>S</u> C QFD <u>S</u> GGPVILRKSQFLVGIISY GM SCAATQY PM <u>G</u> VNTRI
	ere	TGR D <u>S</u> C QFD <u>S</u> GGPVILRKSQFLVGIISY GK SCAETQY PM <u>G</u> VNTRI
	ana	TSR D <u>S</u> C QYD <u>S</u> GGPVILRKSQFLVGIISY GK SCAETS YPM <u>G</u> VNTRV
	pse	NAR D <u>S</u> C QYD <u>S</u> GGPVILRSSKQFLVGIISY GK SCAANAY PM <u>G</u> VNTRI
CG31681	sim	HRW D <u>S</u> C EGD <u>S</u> GGPLIEITTGG- <i>HEL</i> VGIV SW GEGCGTN LG <u>V</u> YEDIA
	mel	QRW D <u>T</u> C QGD <u>S</u> GGPLIETTKGHRQLIGM V SWGDGCGTN PG <u>V</u> YEDIA
	yak	HRG D <u>T</u> C EGD <u>S</u> GGPLVDTKN- - -KKLVGIV SW GVGCGIN PG <u>V</u> YADVA
	ere	PGR SAC <u>R</u> GD <u>S</u> GGPLVEMET- - -RKLIGIV SW GFRCGTAP PG <u>V</u> YADVA
CG32834	sim	~ - - - - - APLVKDGQLVGILSEGG CT TKPDVFAN VP <u>W</u> FTGWI
	mel	NGAG GC SYD T GAPLVIDGQLVGILSEGG CT TKPDVYAN VP <u>W</u> FTGWI
	sec	YGAG GC SYD T GAPLVKDGQLVGILSEGG CT TKPDVFAN VP <u>W</u> FTGWI
	yak	YGAG GC SYD T GAPLVQDDVLVGILSEGG CK TKPDVYAS VG WFKDWI
	ere	YGAG GC SYD T GAALVKDGELVGILSEGG CS TKPDVYAS VA WFKGWI
	ana	SGV GAC SYD T GAPLVKDDKLVGILSEGG CT TPDVYAN VAG FKSWI
	pse	RGT GAC <u>Y</u> LDEGAPLVVNGFLVGNLGT VC AGKPDVYAN LA QHKS W L

Supplementary Table 1: Gene identification numbers for <i>D.simulans</i> and <i>D. melanogaster</i> . <i>D.melanogaster</i> CG and FB gene identification numbers; Function of predicted proteins; Secretion Signal prediction; Transmembrane region prediction; Pairwise d_W/d_S ; PAML data; Presence of orthologs in 12 genomes															
D.sim ID	D.mel id	D.mel CG	D.mel FBgn	Function ¹	SP ²	TM ³	d_W/d_S ⁴	PAML Species ⁵	M0 vs M3		M1 vs M2		M7 vs. M8		12 Genome Species ¹²
									P _s ⁶	d_W/d_S ⁷	P _s ⁸	d_W/d_S ⁹	P _s ¹⁰	d_W/d_S ¹¹	
dsim_GLEANR_16371	CG2233-PA	CG2233	FBgn0029131	Unknown	X		0.95	M.S.Sc.Y.E	0.02	33.31***	0.18	4.16***	0.17	4.37***	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.P	0.23	2.64***	0.10	4.18***	0.17	3.10***	
dsim_GLEANR_3734	CG3066-PA	CG3066	FBgn0042186	Serine Protease	X		0.17	M.S.Sc.Y.E	0.09	1.45***	0.09	1.45	0.09	1.46	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.03	2.56***	0.04	1.00	0.02	2.83*	
								M.S.Sc.Y.E.A.P	0.03	2.63***	0.13	1.00	0.02	2.91	
dsim_GLEANR_9093	CG3831-PA	CG3831	FBgn0033134	Unknown		X	0.32	M.S.Sc.Y.E	0.14	1.54***	0.14	1.54	0.14	1.54	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.23	0.39***	0.01	1.00	0.00	1.00	
								M.S.Sc.Y.E.A.P	0.34	0.20***	0.00	33.13	0.00	1.00	
dsim_GLEANR_7778	Syx5-PA	CG4214	FBgn0033906	Nerve signaling		X	0.36	M.S.Sc.Y.E	0.31	0.54***	0.07	1.00	0.00	1.00	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.30	0.34***	0.04	1.00	0.00	1.00	
								M.S.Sc.Y.E.A.P	0.19	0.46***	0.00	17.20	0.00	1.00	
dsim_GLEANR_8979	tsr-PA	CG4254	FBgn0011726	Actin metabolism			0.00	M.S.Sc.Y.E	0.25	0.00	0.00	1.00	0.00	1.52	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.16	0.00	0.00	1.00	0.00	1.00	
								M.S.Sc.Y.E.A.P	0.02	0.90**	0.00	1.00	0.02	1.00	
dsim_GLEANR_2206	lrf2-PB	CG4370	FBgn0050197	Cation transport		X	0.01	M.S.Sc.Y.E	0.09	1.56***	0.09	1.56	0.09	1.56	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.07	1.85***	0.04	2.21	0.07	1.78	
								M.S.Sc.Y.E.A.P	0.04	2.23***	0.00	5.26	0.03	2.85	
dsim_GLEANR_7402	me31B-PA	CG4916	FBgn0024733	Helicase			0.05	M.S.Sc.Y.E	0.01	2.92	0.01	2.92	0.01	2.92	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.02	0.82***	0.00	1.00	0.02	1.00*	
dsim_GLEANR_2360	DppII-PB	CG7415	FBgn0037580	Peptidase		X	0.01	M.S.Sc.Y.E	0.002	2.92	0.00	2.92	0.002	2.93	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.06	0.67***	0.00	18.57	0.040	1.00*	
								M.S.Sc.Y.E.A.P	0.10	0.44***	0.00	1.00	0.00	1.95	
dsim_GLEANR_10797	Etfalpha48D-PA	CG8280	FBgn0037199	Translation			0.02	M.S.Sc.Y.E	0.00	11.54***	0.00	11.54	0.00	11.54	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.01	2.11***	0.01	2.11	0.01	2.11*	
								M.S.Sc.Y.E.A.P	0.01	3.44***	0.01	3.27*	0.01	3.42***	
dsim_GLEANR_11000	CG8331-PA	CG8331	FBgn0010223	Cell communication		X	0.19	M.S.Sc.Y.E	0.01	33.67***	0.01	34.35	0.01	33.74	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.01	30.56***	0.08	1.00	0.01	30.61*	
								M.S.Sc.Y.E.A.P	0.21	0.59***	0.01	35.52	0.01	30.11**	
dsim_GLEANR_11526	Treh-PC	CG9364	FBgn0035678	Sugar metabolism		X	0.12	M.S.Sc.Y.E	0.05	2.95***	0.04	3.11***	0.05	2.93***	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.04	3.36***	0.02	5.51***	0.03	3.75***	
								M.S.Sc.Y.E.A.P	0.05	2.47***	0.01	5.44	0.03	2.93***	
dsim_GLEANR_11514	Act57B-PA	CG10067	FBgn0052068	Actin formation			0.00	M.S.Y.E.A	0.41	0.00	0.00	1.00	0.00	1.00	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Y.E.A.P	0.37	0.00	0.00	1.00	0.00	1.00	
dsim_GLEANR_13207	CG10469-PA	CG10469	FBgn0052834	Serine Protease		X	0.07	M.S.Sc.Y.E	0.11	0.97	0.04	1.00	0.10	1.00	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.25	0.56***	0.02	1.00	0.05	1.00	
								M.S.Sc.Y.E.A.P	0.21	0.64***	0.04	1.00	0.11	1.00	
dsim_GLEANR_5555	CG10650-PA	CG10650	FBgn0050371	Peptidase		X	0.55	M.S.Sc.Y.A	0.11	2.94***	0.03	4.93	0.06	3.67*	M.S.Sc.Y.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.A.P	0.09	2.97***	0.03	5.36	0.05	3.81**	
dsim_GLEANR_13789	Drs-PA	CG10810	FBgn0010381	Antimicrobial defense		X	0.00	M.S.Sc.Y.E	0.46	0.03	0.00	1.00	0.00	1.00	M.S.Sc.Y.E.A.P.Pr.Mj
								M.S.Sc.Y.E.A	0.32	0.10	0.00	23.07	0.00	1.00	
dsim_GLEANR_12120	CG11137-PA	CG11137	FBgn0029990	Unknown		X	0.10	M.S.Sc.Y.E	0.15	0.24	0.01	1.00	0.00	1.00	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.01	1.80*	0.00	25.23	0.01	1.80*	
								M.S.Sc.Y.E.A.P	0.02	0.84***	0.01	1.00	0.02	1.00*	
dsim_GLEANR_9282	CG11200-PA	CG11200	FBgn0052702	Dehydrogenase		X	0.02	M.S.Sc.Y.E	0.18	0.41	0.04	1.00	0.00	1.00	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.26	0.18*	0.00	36.19	0.00	1.00	
								M.S.Sc.Y.E.A.P	0.06	0.36***	0.00	7.37	0.00	1.00	
dsim_GLEANR_12517	Galpha73B-PA	CG12232	FBgn0039081	Cell communication			0.05	M.S.Sc.Y.E	0.09	0.47***	0.02	1.00	0.00	2.32	M.S.Sc.Y.E.A.P.Pr.W.Mj.V.G
								M.S.Sc.Y.E.A	0.002	9.65***	0.001	10.22	0.002	9.66	
								M.S.Sc.Y.E.A.P	0.03	0.79***	0.001	10.79	0.002	10.24	

Supplementary Table 1: Gene identification numbers for <i>D.simulans</i> and <i>D. melanogaster</i> . D.melanogaster CG and FB gene identification numbers; Function of predicted proteins; Secretion Signal prediction; Transmembrane region prediction; Pairwise d _W d _S ; PAML data; Presence of orthologs in 12 genomes														
D.sim ID	D.mel Id	D.mel CG	D.mel FBgn	Function ¹	SP ²	TM ³	d _W d _S ⁴	PAML Species ⁵	M0 vs M3	M1 vs M2	M7 vs M8	12 Genome Species ¹²		
									P _S ⁶	d _W d _S ⁷	P _S ⁸	d _W d _S ⁹	P _S ¹⁰	d _W d _S ¹¹
dsim_GLEANR_9897	etaTry-PA	CG12386	FBgn0011554	Serine Protease	X		0.14	M.S.Sc.Y.E	0.05	2.35***	0.06	2.09	0.05	2.31
								M.S.Sc.Y.E.A	0.03	3.02***	0.01	3.74	0.03	3.26*
								M.S.Sc.Y.E.A.P	0.03	2.69***	0.07	1.00	0.03	2.65*
dsim_GLEANR_10438	Tsp42El-PA	CG12840	FBgn0039045	Cell communication	X		0.25	M.S.Sc.Y.E	0.005	14.33*	0.00	14.19	0.00	14.22
								M.S.Sc.Y.E.A	0.01	3.83***	0.05	1.00	0.02	3.65
								M.S.Sc.Y.E.A.P	0.15	0.68***	0.03	1.00	0.08	1.00
dsim_GLEANR_9414	CG14495-PA	CG14495	FBgn0034293	Unknown			0.09	M.S.Sc.Y.E	0.09	1.56***	0.09	1.56	0.09	1.56
								M.S.Sc.Y.E.A	0.18	0.81***	0.07	1.00	0.13	1.00
								M.S.Sc.Y.E.A.P	0.01	2.66***	0.00	16.63	0.00	3.18
dsim_GLEANR_9351	CG15098-PA	CG15098	FBgn0037580	Unknown	X		0.69	M.S.Sc.Y.E	0.02	4.63***	0.04	3.84	0.05	3.23
								M.S.Sc.Y.E.A	0.09	2.77***	0.04	3.66	0.07	3.00*
								M.S.Sc.Y.E.A.P	0.09	2.72***	0.05	3.46	0.08	2.89***
dsim_GLEANR_9344	Jheh3-PA	CG15106	FBgn0034406	Juv. Horm. Catabolism	X	X	0.06	M.S.Sc.Y.E	0.15	0.48***	0.03	1.00	0.00	1.00
								M.S.Sc.Y.E.A	0.19	0.37***	0.00	38.74	0.01	1.00
								M.S.Sc.Y.E.A.P	0.15	0.22***	0.03	1.00	0.00	1.47
dsim_GLEANR_5780	CG15293-PA	CG15293	FBgn0051343	Unknown	X		0.49	M.S.Sc.Y.E	0.20	1.42***	0.07	1.69	0.49	1.15
								M.S.Sc.Y.E.A	0.08	2.29***	0.04	1.00	0.03	2.82
								M.S.Sc.Y.E.A.P	0.07	2.32***	0.15	1.00	0.05	2.41
dsim_GLEANR_5271	CG15533-PA	CG15533	FBgn0053306	Phospholipid metabolism	X	X	0.13	M.S.Sc.Y.E	0.19	0.47***	0.05	1.00	0.00	1.00
								M.S.Sc.Y.E.A	0.19	0.47***	0.00	21.49	0.00	1.00
								M.S.Sc.Y.E.A.P	0.01	1.08***	0.01	1.00	0.00	1.11
dsim_GLEANR_6593	CG17012-PA	CG17012	FBgn0051686	Serine Protease	X		0.89	M.S.Sc.A	0.07	6.08***	0.05	8.21***	0.06	6.96***
dsim_GLEANR_2235	CG17119-PA	CG17119	FBgn0042187	Amino acid transport	X	X	0.07	M.S.Sc.Y.E	0.04	1.54***	0.04	1.54	0.04	1.55
								M.S.Sc.Y.E.A	0.08	1.02***	0.00	0.03	0.07	1.00
								M.S.Sc.Y.E.A.P	0.08	0.98***	0.00	2.44	0.06	1.06
dsim_GLEANR_6882	CG17234-PA	CG17234	FBgn0044419	Serine Protease	X		0.74	M.S.Sc.Y.E	0.05	7.23***	0.09	5.25***	0.10	4.86***
								M.S.Sc.Y.E.P	0.06	5.94***	0.10	4.20***	0.20	2.82***
dsim_GLEANR_6595	CG17239-PA	CG17239	FBgn0042186	Serine Protease	X		0.73	M.S.Sc.Y.E	0.22	2.40***	0.22	2.41*	0.22	2.40*
								M.S.Sc.Y.E.A	0.16	2.71***	0.09	3.41***	0.13	2.94***
dsim_GLEANR_6596	Ser12-PA	CG17240	FBgn0011708	Serine Protease	X		0.64	M.S.Y.E	0.09	6.27***	0.09	6.25***	0.09	6.25***
dsim_GLEANR_12116	Qm-PA	CG17521	FBgn0024733	Translation			0.02	M.S.Sc.Y.E	0.005	2.77**	0.01	2.77	0.00	2.78*
								M.S.Sc.Y.E.A	0.005	3.25***	0.01	3.25	0.01	3.25**
								M.S.Sc.Y.E.A.P	0.01	1.75***	0.00	1.69	0.01	1.75*
dsim_GLEANR_5775	CG18125-PA	CG18125	FBgn0011726	Serine Protease	X		0.79							M.S.Sc
dsim_GLEANR_11034	CG30197-PA	CG30197	FBgn0050197	Unknown	X		0.05	M.S.Sc.Y.E	0.02	4.37***	0.02	4.37	0.002	4.37*
								M.S.Sc.Y.E.A	0.22	0.28*	0.00	1.00	0.000	2.90
								M.S.Sc.Y.E.A.P	0.33	0.16	0.00	1.00	0.004	4.26
dsim_GLEANR_15401	CG30371-PA	CG30371	FBgn0034500	Serine Protease	X		0.13	M.S.Sc.Y.E	0.36	0.48*	0.05	1.00	1.05	1.00
								M.S.Sc.Y.E.A	0.02	1.35***	0.04	1.00	0.003	1.57
								M.S.Sc.Y.E.A.P	0.02	1.40***	0.00	27.59	0.01	1.75
dsim_GLEANR_4658	CG31343-PA	CG31343	FBgn0051343	Peptidase		X	0.14	M.S.Sc.Y.E	0.13	1.11***	0.13	1.11	0.12	1.12
								M.S.Sc.Y.E.A	0.06	1.32***	0.19	1.00	0.02	1.91
								M.S.Sc.Y.E.A.P	0.04	1.37***	0.04	1.00	0.01	2.02
dsim_GLEANR_18113	CG31681-PA	CG31681	FBgn0034406	Serine Protease	X		0.82	M.S.Y.E	0.09	5.57***	0.08	6.05***	0.09	5.84***
dsim_GLEANR_6881	CG31686-PA	CG31686	FBgn0034398	Unknown			0.66							M.S.Sc.Y.E
dsim_GLEANR_14267	CG32068-PA	CG32068	FBgn0034293	Secondary metabolism			0.39	M.S.Sc.Y.E	0.08	1.18***	0.08	1.18	0.08	1.18
								M.S.Sc.Y.E.A	0.02	0.92***	0.01	1.00	0.00	1.00
								M.S.Sc.Y.E.A.P	0.33	0.20***	0.00	5.32	0.00	1.23

Supplementary Table 1: Gene identification numbers for <i>D.simulans</i> and <i>D. melanogaster</i> , D.melanogaster CG and FB gene identification numbers; Function of predicted proteins; Secretion Signal prediction; Transmembrane region prediction; Pairwise d_N/d_S ; PAML data; Presence of orthologs in 12 genomes														
D.sim ID	D.mel id	D.mel CG	D.mel FBgn	Function ¹	SP ²	TM ³	d_N/d_S ⁴	PAML Species ⁵	M0 vs M3		M1 vs M2		M7 vs. M8	
									P _s ⁶	d_N/d_S ⁷	P _s ⁸	d_N/d_S ⁹	P _s ¹⁰	d_N/d_S ¹¹
dsim_GLEANR_17130	CG32702-PA	CG32072	FBgn0011554	Protein-protein interact.			0.10	M.S.Sc.Y.E	0.02	2.55***	0.00	6.09	0.01	3.58**
								M.S.Sc.Y.E.P	0.06	1.41***	0.05	1.00	0.03	2.11***
dsim_GLEANR_15288	CG32834-PA	CG32834	FBgn0052834	Serine Protease	X		0.19	M.S.Sc.Y.E	0.21	1.76***	0.21	1.76	0.20	1.77
								M.S.Sc.Y.E.A	0.08	2.75***	0.02	4.94	0.04	3.47
								M.S.Sc.Y.E.A.P	0.09	2.74***	0.02	5.15	0.06	3.25*
dsim_GLEANR_10398	debd-PA	CG33134	FBgn0051343	Apoptosis	X	X	0.06	M.S.Sc.Y.E	0.06	0.97	0.03	1.00	0.06	1.00
								M.S.Sc.Y.E.A	0.06	0.86***	0.00	28.21	0.06	1.00
								M.S.Sc.Y.E.A.P	0.06	0.71***	0.00	2.11	0.04	1.00
dsim_GLEANR_5795	CG33306-PA	CG33306	FBgn0003748	Unknown	X		0.04	M.S.Sc.Y.E	0.27	0.47	0.03	1.00	0.00	1.00
								M.S.Sc.Y.E.A	0.11	0.74***	0.05	1.00	0.03	1.00
								M.S.Sc.Y.E.A.P	0.37	0.40***	0.05	1.00	0.02	1.75
dsim_GLEANR_15604	no mel													
dsim_GLEANR_6594	no mel													

¹Predicted protein function; ²"X" indicates that the proteins has a predicted secretion signal; ³"X" indicates the proteins has a predicted transmembrane region; ⁴ d_N/d_S : pairwise comparison of *D. melanogaster* and *D. simulans* sequences, estimated assuming no rate heterogeneity; ⁵Species of *Drosophila* from which sequences were obtained for PAML analysis M= *melanogaster*, S = *simulans*, Sc= *sechellia*, Y = *yakuba*, E = *erecta*; A= *ananassae*; P = *pseudobscura*. The following statistics are all derived from PAML analysis: ⁶P_s: the proportion of sites estimated to belong to the class that has the highest d_N/d_S in M3; ⁷ d_N/d_S : for the highest class in M3; ⁸P_s: the proportion of sites estimated to belong to the class that has $d_N/d_S > 1$ in M2; ⁹ d_N/d_S : the estimate for the class with the ratio > 1 in M2; ¹⁰P_s: the proportion of sites estimated to belong to the class that has $d_N/d_S > 1$ in M8; ¹¹ d_N/d_S : the estimate for the class with the ratio > 1 in M8; *P < 0.05; **P < 0.01; ***P < 0.001. ¹²The genomes in which orthologs were found using the 12 *Drosophila* genomes. Pr = *persimilis*, W = *willistoni*, Mj = *mojavensis*, V = *virilis*, and G = *grimshawi*

[illegible][illegible]

Supplementary Table 2: Gene identification numbers for <i>D.simulans</i> and <i>D. melanogaster</i> ; Pairwise d _w /d _s ; PAML branch model data.																																								
dsim ID	dmel ID	dn/ds ¹	Tree ²	lnL ³	branch ⁴	dn ⁵	ds ⁶	dn/ds ⁷	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds				
dsim_GLEANR_16371	CG2233-PA	0.95	(Y,E,(M,(S,Sc)));	-1836.93	6..7	0.056	0.000	999.000	7..2	0.026	0.076	0.340	7..8	0.017	0.038	0.453	8..1	0.011	0.000	999.000	8..3	0.011	0.007	1.702	6..9	0.000	0.152	0.000	9..4	0.073	0.104	0.702	9..5	0.093	0.111	0.835				
dsim_GLEANR_3734	CG3066-PA	0.17	(A,(M,(S,Sc))),(Y,E));	-3463.06	7..6	0.153	1.882	0.082	7..8	0.008	0.109	0.074	8..2	0.011	0.062	0.179	8..9	0.009	0.023	0.399	9..1	0.000	0.032	0.000	9..3	0.003	0.007	0.487	7..10	0.005	0.000	773.274	10..4	0.017	0.081	0.207	10..5	0.019	0.170	0.113
			(A,E,(Y,(M,(S,Sc))));	-3458.82	7..6	0.149	1.934	0.077	7..4	0.010	0.082	0.119	7..8	0.008	0.000	999.000	8..5	0.019	0.170	0.113	8..9	0.012	0.109	0.108	9..2	0.011	0.062	0.176	9..10	0.009	0.023	0.399	10..1	0.000	0.032	0.000	10..3	0.003	0.007	0.486
			(A,Y,(E,(M,(S,Sc))));	-3463.77	7..6	0.154	1.924	0.080	7..5	0.016	0.170	0.093	7..8	0.005	0.000	153.217	8..4	0.017	0.081	0.210	8..9	0.012	0.109	0.111	9..2	0.011	0.062	0.176	9..10	0.009	0.023	0.408	10..1	0.000	0.032	0.000	10..3	0.003	0.007	0.489
dsim_GLEANR_9093	CG3831-PA	0.32	(A,(M,(S,Sc))),(Y,E));	-3282.23	7..6	0.030	1.324	0.023	7..8	0.001	0.070	0.016	8..2	0.002	0.046	0.047	8..9	0.001	0.033	0.033	9..1	0.064	0.089	0.718	9..3	0.001	0.025	0.045	7..10	0.001	0.034	0.029	10..4	0.004	0.077	0.057	10..5	0.006	0.068	0.080
			(A,E,(Y,(M,(S,Sc))));	-3284.09	7..6	0.030	1.371	0.022	7..4	0.005	0.080	0.056	7..8	0.000	0.000	0.518	8..5	0.006	0.068	0.091	8..9	0.001	0.103	0.013	9..2	0.002	0.046	0.047	9..10	0.001	0.033	0.032	10..1	0.064	0.090	0.712	10..3	0.001	0.024	0.046
			(A,Y,(E,(M,(S,Sc))));	-3282.31	7..6	0.029	1.359	0.021	7..5	0.005	0.055	0.091	7..8	0.001	0.014	0.088	8..4	0.004	0.080	0.056	8..9	0.001	0.103	0.013	9..2	0.002	0.046	0.047	9..10	0.001	0.033	0.032	10..1	0.064	0.090	0.711	10..3	0.001	0.024	0.046
dsim_GLEANR_7778	Syx5-PA	0.36	(A,(M,(S,Sc))),(Y,E));	-3715.15	7..6	0.057	1.242	0.046	7..8	0.000	0.072	0.000	8..2	0.002	0.042	0.036	8..9	0.004	0.026	0.137	9..1	0.039	0.070	0.560	9..3	0.003	0.019	0.153	7..10	0.003	0.042	0.061	10..4	0.011	0.113	0.095	10..5	0.013	0.098	0.130
			(A,E,(Y,(M,(S,Sc))));	-3720.43	7..6	0.057	1.273	0.045	7..4	0.011	0.113	0.096	7..8	0.001	0.000	555.483	8..5	0.013	0.098	0.137	8..9	0.002	0.114	0.017	9..2	0.001	0.042	0.033	9..10	0.004	0.026	0.138	10..1	0.039	0.071	0.559	10..3	0.003	0.019	0.153
			(A,Y,(E,(M,(S,Sc))));	-3717.23	7..6	0.056	1.268	0.044	7..5	0.011	0.092	0.116	7..8	0.003	0.006	0.493	8..4	0.012	0.113	0.102	8..9	0.002	0.114	0.017	9..2	0.001	0.042	0.033	9..10	0.004	0.026	0.139	10..1	0.040	0.070	0.561	10..3	0.003	0.019	0.154
dsim_GLEANR_8979	tsr-PA	0.00	(A,(M,(S,Sc))),(Y,E));	-748.12	7..6	0.000	0.464	0.000	7..8	0.000	0.026	0.000	8..2	0.000	0.046	0.000	8..9	0.000	0.000	0.309	9..1	0.000	0.000	0.000	9..3	0.000	0.000	0.000	7..10	0.000	0.021	0.000	10..4	0.000	0.035	0.000	10..5	0.000	0.012	0.000
			(A,E,(Y,(M,(S,Sc))));	-748.40	7..6	0.000	0.489	0.000	7..4	0.000	0.022	0.000	7..8	0.000	0.013	0.000	8..5	0.000	0.012	0.000	8..9	0.000	0.048	0.000	9..2	0.000	0.047	0.000	9..10	0.000	0.000	0.416	10..1	0.000	0.000	0.000	10..3	0.000	0.000	0.325
			(A,Y,(E,(M,(S,Sc))));	-749.03	7..6	0.000	0.495	0.000	7..5	0.000	0.012	0.000	7..8	0.000	0.000	0.707	8..4	0.000	0.035	0.000	8..9	0.000	0.047	0.000	9..2	0.000	0.047	0.000	9..10	0.000	0.000	0.452	10..1	0.000	0.000	0.000	10..3	0.000	0.000	0.000
dsim_GLEANR_2206	Irk2-PB	0.01	(A,(M,(S,Sc))),(Y,E));	-2385.15	7..6	0.008	1.176	0.007	7..8	0.001	0.095	0.013	8..2	0.001	0.069	0.017	8..9	0.000	0.009	0.000	9..1	0.000	0.039	0.000	9..3	0.000	0.035	0.000	7..10	0.000	0.000	1.061	10..4	0.000	0.138	0.000	10..5	0.000	0.089	0.000
			(A,E,(Y,(M,(S,Sc))));	-2383.03	7..6	0.008	1.118	0.007	7..4	0.000	0.068	0.000	7..8	0.000	0.071	0.000	8..5	0.000	0.087	0.000	8..9	0.001	0.094	0.013	9..2	0.001	0.068	0.018	9..10	0.000	0.009	0.000	10..1	0.000	0.039	0.000	10..3	0.000	0.035	0.000
			(A,Y,(E,(M,(S,Sc))));	-2385.15	7..6	0.008	1.176	0.007	7..5	0.000	0.089	0.000	7..8	0.000	0.000	3.147	8..4	0.000	0.138	0.000	8..9	0.001	0.095	0.013	9..2	0.001	0.069	0.017	9..10	0.000	0.009	0.000	10..1	0.000	0.039	0.000	10..3	0.000	0.035	0.000
dsim_GLEANR_7402	me31B-PA	0.05	(A,(M,(S,Sc))),(Y,E));	-2630.69	7..6	0.007	0.580	0.012	7..8	0.000	0.034	0.000	8..2	0.000	0.022	0.000	8..9	0.002	0.012	0.164	9..1	0.000	0.009	0.000	9..3	0.000	0.012	0.000	7..10	0.001	0.025	0.039	10..4	0.000	0.032	0.000	10..5	0.000	0.038	0.000
			(A,E,(Y,(M,(S,Sc))));	-2639.49	7..6	0.008	0.608	0.013	7..4	0.000	0.033	0.000	7..8	0.000	0.000	17.244	8..5	0.000	0.038	0.000	8..9	0.001	0.058	0.017	9..2	0.000	0.022	0.000	9..10	0.002	0.012	0.163	10..1	0.000	0.009	0.000	10..3	0.000	0.012	0.000
			(A,Y,(E,(M,(S,Sc))));	-2639.49	7..6	0.008	0.608	0.013	7..5	0.000	0.038	0.000	7..8	0.000	0.000	0.344	8..4	0.000	0.033	0.000	8..9	0.001	0.058	0.017	9..2	0.000	0.022	0.000	9..10	0.002	0.012	0.163	10..1	0.000	0.009	0.000	10..3	0.000	0.012	0.000
dsim_GLEANR_2360	DppIII-PB	0.01	(A,(M,(S,Sc))),(Y,E));	-5012.71	7..6	0.046	2.023	0.023	7..8	0.005	0.067	0.082	8..2	0.001	0.074	0.016	8..9	0.000	0.047	0.000	9..1	0.000	0.009	0.000	9..3	0.001	0.031	0.037	7..10	0.001	0.024	0.031	10..4	0.006	0.083	0.068	10..5	0.004	0.127	0.035
			(A,E,(Y,(M,(S,Sc))));	-4995.93	7..6	0.043	2.017	0.021	7..4	0.002	0.062	0.038	7..8	0.004	0.021	0.170	8..5	0.004	0.127	0.033	8..9	0.006	0.090	0.066	9..2	0.001	0.075	0.016	9..10	0.000	0.047	0.000	10..1	0.000	0.009	0.000	10..3	0.001	0.031	0.037
			(A,Y,(E,(M,(S,Sc))));																																					

Supplementary Table 2: Gene identification numbers for <i>D.simulans</i> and <i>D. melanogaster</i> ; Pairwise d _N /d _S ; PAML branch model data.																																								
dsim ID	dmel ID	dn/ds ¹	Tree ²	lnL ³	branch ⁴	dn ⁵	ds ⁶	dn/ds ⁷	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds	branch	dn	ds	dn/ds				
dsim_GLEANR_6593	CG17012-PA	0.89																																						
dsim_GLEANR_2235	CG17119-PA	0.07	(A,(M,(S,Sc))),(Y,E));	-3205.82	7..6	0.100	1.432	0.070	7..8	0.007	0.060	0.116	8..2	0.010	0.119	0.083	8..9	0.003	0.034	0.091	9..1	0.001	0.065	0.021	9..3	0.003	0.025	0.126	7..10	0.001	0.061	0.021	10..4	0.015	0.112	0.131	10..5	0.010	0.073	0.139
			(A,E,(Y,(M,(S,Sc)))));	-3203.70	7..6	0.097	1.470	0.066	7..4	0.012	0.072	0.162	7..8	0.004	0.042	0.095	8..5	0.009	0.073	0.130	8..9	0.008	0.120	0.064	9..2	0.010	0.121	0.085	9..10	0.003	0.033	0.080	10..1	0.002	0.065	0.023	10..3	0.003	0.025	0.121
			(A,Y,(E,(M,(S,Sc)))));	-3206.81	7..6	0.100	1.502	0.066	7..5	0.009	0.074	0.121	7..8	0.001	0.000	998.864	8..4	0.015	0.112	0.131	8..9	0.008	0.120	0.066	9..2	0.010	0.120	0.082	9..10	0.003	0.033	0.095	10..1	0.001	0.065	0.020	10..3	0.003	0.025	0.128
dsim_GLEANR_6882	CG17234-PA	0.74	(Y,E,(M,(S,Sc)))	-2205.88	6..7	0.049	0.088	0.561	7..2	0.028	0.032	0.876	7..8	0.033	0.027	1.224	8..1	0.002	0.041	0.049	8..3	0.014	0.012	1.112	6..5	0.193	0.292	0.659	6..4	0.094	0.126	0.749								
dsim_GLEANR_6595	CG17239-PA	0.73	(A,(M,(S,Sc))),(Y,E));	-2543.84	7..6	0.274	1.097	0.250	7..8	0.046	0.141	0.328	8..2	0.048	0.045	1.057	8..9	0.025	0.051	0.486	9..1	0.013	0.018	0.710	9..3	0.004	0.030	0.123	7..10	0.027	0.006	4.743	10..4	0.074	0.175	0.423	10..5	0.040	0.041	0.971
			(A,E,(Y,(M,(S,Sc)))));	-2549.15	7..6	0.284	1.124	0.253	7..4	0.070	0.174	0.401	7..8	0.007	0.000	999.000	8..5	0.040	0.040	0.997	8..9	0.070	0.148	0.473	9..2	0.047	0.045	1.052	9..10	0.025	0.051	0.482	10..1	0.012	0.018	0.648	10..3	0.005	0.030	0.166
			(A,Y,(E,(M,(S,Sc)))));	-2548.01	7..6	0.285	1.119	0.255	7..5	0.033	0.031	1.076	7..8	0.011	0.014	0.752	8..4	0.073	0.172	0.422	8..9	0.070	0.143	0.488	9..2	0.047	0.045	1.038	9..10	0.024	0.051	0.474	10..1	0.011	0.018	0.633	10..3	0.005	0.030	0.174
dsim_GLEANR_6596	Ser12-PA	0.64	(Y,E,(M,S))	-2085.49	5..3	1.089	0.101	0.093	5..4	0.451	0.114	0.252	5..6	0.661	0.040	0.060	6..2	0.508	0.033	0.066	6..1	0.723	0.038	0.053																
dsim_GLEANR_12116	Qm-PA	0.02	(A,(M,(S,Sc))),(Y,E));	-1391.59	7..6	0.008	1.044	0.008	7..8	0.000	0.047	0.000	8..2	0.002	0.044	0.052	8..9	0.000	0.042	0.000	9..1	0.000	0.029	0.000	9..3	0.000	0.007	0.000	7..10	0.000	0.029	0.000	10..4	0.000	0.154	0.000	10..5	0.002	0.081	0.025
			(A,E,(Y,(M,(S,Sc)))));	-1392.29	7..6	0.008	1.056	0.008	7..4	0.000	0.156	0.000	7..8	0.000	0.000	0.439	8..5	0.002	0.083	0.024	8..9	0.000	0.073	0.000	9..2	0.002	0.044	0.053	9..10	0.000	0.042	0.000	10..1	0.000	0.028	0.000	10..3	0.000	0.007	0.000
			(A,Y,(E,(M,(S,Sc)))));	-1390.90	7..6	0.008	1.042	0.008	7..5	0.000	0.082	0.000	7..8	0.002	0.000	999.000	8..4	0.000	0.155	0.000	8..9	0.000	0.072	0.000	9..2	0.002	0.044	0.053	9..10	0.000	0.042	0.000	10..1	0.000	0.028	0.000	10..3	0.000	0.007	0.000
dsim_GLEANR_5775	CG18125-PA	0.79																																						
dsim_GLEANR_11034	CG30197-PA	0.05	(A,(M,(S,Sc))),(Y,E));	-636.80	7..6	0.056	1.179	0.048	7..8	0.000	0.088	0.000	8..2	0.000	0.099	0.000	8..9	0.005	0.000	96.765	9..1	0.000	0.000	1.807	9..3	0.013	0.001	12.117	7..10	0.000	0.085	0.000	10..4	0.000	0.079	0.000	10..5	0.005	0.102	0.045
			(A,E,(Y,(M,(S,Sc)))));	-638.67	7..6	0.056	1.266	0.044	7..4	0.000	0.052	0.000	7..8	0.000	0.030	0.000	8..5	0.005	0.102	0.045	8..9	0.000	0.172	0.000	9..2	0.000	0.099	0.000	9..10	0.005	0.000	664.372	10..1	0.000	0.000	0.000	10..3	0.014	0.000	33.173
			(A,Y,(E,(M,(S,Sc)))));	-637.93	7..6	0.056	1.293	0.043	7..5	0.005	0.000	117.679	7..8	0.000	0.101	0.000	8..4	0.000	0.079	0.000	8..9	0.000	0.171	0.000	9..2	0.000	0.098	0.000	9..10	0.005	0.000	999.000	10..1	0.000	0.000	0.145	10..3	0.014	0.001	16.774
dsim_GLEANR_15401	CG30371-PA	0.13	(A,(M,(S,Sc))),(Y,E));	-3288.81	7..6	0.089	1.150	0.077	7..8	0.007	0.051	0.144	8..2	0.008	0.062	0.135	8..9	0.005	0.042	0.127	9..1	0.001	0.020	0.043	9..3	0.004	0.018	0.202	7..10	0.001	0.083	0.014	10..4	0.030	0.092	0.325	10..5	0.024	0.115	0.206
			(A,E,(Y,(M,(S,Sc)))));	-3285.11	7..6	0.084	1.252	0.067	7..4	0.024	0.093	0.258	7..8	0.008	0.000	999.000	8..5	0.023	0.115	0.202	8..9	0.007	0.133	0.052	9..2	0.008	0.061	0.133	9..10	0.005	0.042	0.127	10..1	0.001	0.019	0.056	10..3	0.003	0.018	0.188
			(A,Y,(E,(M,(S,Sc)))));	-3283.11	7..6	0.085	1.208	0.070	7..5	0.019	0.044	0.434	7..8	0.006	0.074	0.079	8..4	0.030	0.091	0.326	8..9	0.007	0.131	0.055	9..2	0.009	0.060	0.142	9..10	0.005	0.042	0.122	10..1	0.001	0.019	0.046	10..3	0.004	0.018	0.201
dsim_GLEANR_4658	CG31343-PA	0.14	(A,(M,(S,Sc))),(Y,E));	-8749.10	7..6	0.165	1.880	0.088	7..8	0.008	0.055	0.152	8..2	0.010	0.091	0.112	8..9	0.006	0.030	0.199	9..1	0.001	0.016	0.064	9..3	0.005	0.035	0.127	7..10	0.005	0.041	0.119	10..4	0.026	0.154	0.172	10..5	0.042	0.184	0.228
			(A,E,(Y,(M,(S,Sc)))));	-8747.39	7..6	0.162	1.871	0.087	7..4	0.022	0.108	0.204	7..8	0.006	0.046	0.130	8..5	0.042	0.185	0.229	8..9	0.012	0.094	0.123	9..2	0.011	0.091	0.115	9..10	0.006	0.030	0.193	10..1	0.001	0.016	0.071	10..3	0.004	0.035	0.125
			(A,Y,(E,(M,(S,Sc)))));	-8721.09	7..6	0.153	1.913	0.080	7..5	0.027	0.166	0.164	7..8	0.017	0.020	0.833	8..4	0.027	0.153	0.173	8..9	0.011	0.095	0.119	9..2	0.010	0.091	0.113	9..10	0.006	0.030	0.198	10..1	0.001	0.016	0.070	10..3	0.004	0.035	0.125
dsim_GLEANR_18113	CG31681-PA	0.82	(Y,E,(M,S))	-2199.12	5..3	0.413	0.072	0.175	5..4	0.606	0.094	0.154	5..6	0.411	0.070	0.171	6..2	0.873	0.062	0.072	6..1	0.714	0.044	0.062																
dsim_GLEANR_6881	CG31686-PA	0.66																																						
dsim_GLEANR_14267	CG32068-PA	0.39	(A,(M,(S,Sc))),(Y,E));	-1253.39	7..6	0.055	1.924	0.029	7..8	0.003	0.038	0.069	8..2	0.069	0.181	0.381	8..9	0.000	0.017	0.000	9..1	0.000	0.000	0.000	9..3	0.000	0.070	0.000	7..10	0.000	0.000	0.648	10..4	0.003	0.193	0.013	10..5	0.005	0.145	0.036
			(A,E,(Y,(M,(S,Sc)))));	-1253.35	7..6	0.055	1.894	0.029	7..4	0.003	0.166	0.016	7..8	0.000	0.027	0.000	8..5	0.005	0.145	0.036	8..9	0.003	0.038	0.069	9..2	0.069	0.181	0.380	9..10	0.000	0.017	0.000	10..1	0.000	0.000	0.000	10..3	0.000	0.070	0.000
			(A,Y,(E,(M,(S,Sc)))));	-1253.38	7..6	0.055	1.922	0.029	7..5	0.005	0.135	0.039	7..8	0.000	0.010	0.000	8..4	0.003	0.194	0.013	8..9	0.003	0.038	0.069	9..2	0.069	0.181	0.380	9..10	0.000	0.017	0.000	10..1	0.000	0.000	0.000	10..3	0.000		